

THE EFFECT OF ULTRASOUND ON THE POPULATION  
GROWTH OF EUGLENA GRACILIS

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The Graduate Division  
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Master of Arts

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by  
Gerald Edwin Dunn

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## CHAPTER I

### INTRODUCTION

Microorganisms are widely used in many irradiation experiments. They possess desirable characteristics for this research because they can be maintained in sterile culture, because of the rapid growth of a population, and because of the relative ease with which they may be counted. The methodology used may be varied greatly and many environmental conditions can be studied in short periods of time.

Microorganisms exhibit the same growth patterns as do higher organisms. Yet the unicellular organism tends to be affected in different ways. Small changes in the environment may induce large changes in the growth pattern.

The protozoan, Euglena, has characteristics of both plants and animals. Because of the presence of chlorophyll and other chromatophores, Euglena is often classed as an autotroph. In its mobility and at times heterotrophic metabolism, it exhibits properties of the typical animal.

Euglena has been studied using different types of irradiation, ultraviolet, radio active cobalt, and X-ray. The effect of physical wave action on the organism has received less attention. The effect of ultrasound on Euglena would shed light on the influence of another source of

energy, one which is used in industry and in some homes. Studies on microorganisms may have significance when the information is applied to higher forms.

The purpose of this study was: to (1) develop methods for the determination of the effect of ultrasonics on the population growth of Euglena; and (2) to describe its effect.

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## CHAPTER II

### REVIEW OF THE LITERATURE

Ultrasonic vibrations act upon cells in many different ways and these actions may cause population differences. Goldman and Lepeschkin, working with the algae Spiragyra, noted that cavitation acted as an external source of shock waves which produced injury by steadily bombarding the cells.<sup>1</sup>

Victor T. Tomberg has indicated that studies at Brussels (1946-1952) showed that cavitation depends on the viscosity of the irradiated material and the presence of gas bubbles inside such matter. Gas bubbles have certain weaknesses in withstanding ultrasonic waves. They vibrate and reach a critical diameter at which point they burst, forming hollow spaces of low gas pressure.<sup>2</sup>

Harvey and Loomis, as cited by Gordon, found that very low intensity of ultrasound caused a micro-stirring of cellular protoplasm. The stirring action increased with increased

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<sup>1</sup>D. E. Goldman and W. W. Lepeschkin, "Injury to Living Cells in Standing Sound Waves," Journal of Cellular and Comparative Physiology, XL (October, 1952), 255.

<sup>2</sup>Victor T. Tomberg, "Ultrasonic Daismetry for Medical and Biological Work," Ultrasonic News, I (February, 1958), 24.

intensity until chloroplasts were disoriented, protoplasm constituents became separated from each other, and cells were finally ruptured which caused death.<sup>1</sup>

After irradiation with ultrasonic vibrations the protoplasm of the yeast Saccharomycetes cerevisiae was changed in appearance. The spherical vacuoles took on irregular shapes; the protoplasm congealed, contracted, and drew away from the cell walls.<sup>2</sup>

The physical pressures of the sound waves are the most obvious means of cell destruction. Some researchers have suggested less obvious methods for explaining the effects upon cells. Wood stated that one of the most surprising and spectacular effects produced by supersonic waves traveling through a liquid is the aggregation of minute suspended particles into a single lump. This is associated in some way with the presence of dissolved air in the water. The use of moderate intensities rendered small unicellular organisms such as Paramecium immobile for a period of time.<sup>3</sup>

Newcomer and Wallace discovered that the primary physiological effect of X-ray and mustard gas in producing

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<sup>1</sup>Andrew G. Gordon, "The Use of Ultrasonics in Agriculture," Ultrasonics, I (April-June, 1963), 70.

<sup>2</sup>Goldman and Lepschkin, op. cit., p. 383.

<sup>3</sup>Robert William Woods, Supersonics (Providence, Rhode Island: Brown University Press, 1939), p. 73.

adhesiveness of the dividing chromosomes and chromatic breakage were produced simultaneously by ultrasonic vibrations.<sup>1</sup>

Newcomer found that sound waves of sufficient intensity to produce visible nuclear or chromosomal aberrations in the root tip of Narcissus poeticus, were apparently lethal, and no new chromosomal reorganizations were observed.<sup>2</sup>

Death of microorganisms and of animal and vegetable cells is not the only effect of ultrasonic waves. Fadeeva and El'piner reported there are indications that functional and physiocolloidal disruptions of cell structure which do not lead to the death of the cells were caused by ultrasonic energy. It was established that the electrokinetic potential of a cell also undergoes certain changes in the field of ultrasonic waves.<sup>3</sup>

Loza, as cited by Gordon, states it is a long known fact that diffusion and osmosis are speeded up by ultrasound and more recently that ultrasonic changes the chemical

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<sup>1</sup>Earl H. Newcomer and R. H. Wallace, "Chromosomal and Nuclear Aberrations Induced by Ultrasonic Vibrations," American Journal of Botany, XXXVI (January-June, 1954), 231.

<sup>2</sup>Earl Newcomer, "Observations on Dosage, the Mechanism of Action and the Recovery of Cells Exposed to Ultrasonic Vibrations," American Journal of Botany, XLI (January-June, 1954), 384-86.

<sup>3</sup>M. P. Fadeeva and I. E. El'piner, "The Effect of Ultrasonic Waves on Asotobacter Chroococcum," Microbiology, XXVIII (July-August, 1959), 458.

selectivity of semi-permeable membranes.<sup>1</sup> This, of course, affects materials which enter and leave the cell--materials so necessary for the life of the cell.

Istomina and Ostrousky, as cited by Gordon, believe that all plant biochemical reactions in potato growth are governed by enzymes which were found in many cases to be influenced by ultrasound.<sup>2</sup>

Topa, while working with ultrasonic waves on antibiotic producing fungi, concluded that the action of an acoustic field on spores of mycelia of Penicillium, Chrysogenum, and Streptomyces brings about permanent morphological and functional changes. He concluded further that in this new manner, new antibiotics could be obtained and that the present output of these products could be increased.<sup>3</sup>

With ultrasonic action there is also a raise in temperature of the organism and the medium in which the organism lives. Crawford, while working with ultrasonics and the roach Periplaneta americana, reported a two inch long insect was killed quickly by a fast rise in the internal temperature.<sup>4</sup>

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<sup>1</sup>Gordon, op. cit., p. 70.

<sup>2</sup>Ibid., p. 72.

<sup>3</sup>Mieczyslaw Topa, "Effect of Ultrasonic Waves on Antibiotic Fungi," The Ultrasonic News, V (Summer, 1961), 4.

<sup>4</sup>Alan E. Crawford, Ultrasonic Engineering (New York: Academic Press, Inc., 1955), p. 287.

Obalesky as cited by Gordon states temperature increase of treated organisms has been known as one of the more spectacular effects of ultrasonics. Heat may be responsible for a temporary acceleration of some bio-chemical reactions, and lethal effects on organisms.<sup>1</sup>

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<sup>1</sup>Gordon, op. cit., p. 72.



### CHAPTER III

#### MATERIALS AND METHODS

A bacteria free culture of Euglena gracilis was obtained from the National Biological Supply Company, Chicago, Illinois. Sterile transfers were made to Euglena broth 30690-01 from Difco Laboratories. Young cultures were maintained throughout the experiment, using the sterile transfer technique.

The number of organisms per unit volume was determined with the aid of a densimeter designed and constructed for this purpose. A beam of light was passed through the culture tube on to a photoelectric cell. The resistance of the circuit was read on an Eico Model 249 vacuum tube Electronic voltmeter ohmmeter. The ohmite range was from 1 to 1000 ohms of resistance.

Each culture tube was tested with only Euglena broth and marked as to the correct position in the densimeter for the least absorption. Twenty, one hundred fifty ml. glass test tubes with the same transmission index were used throughout the experiment. Each test tube was inserted into the densimeter with caution so as to maintain it in the proper position.

Test tubes containing twenty five ml. of sterilized

Euglena medium were inoculated. Each day the culture density of Euglena gracilis was determined with the aid of the densimeter.

A hemocytometer was used to determine the number of Euglena per unit volume and in turn correlated with the density ohmite reading. One-tenth of a cubic millimeter of a given concentration of Euglena was placed on the hemocytometer and photographed under a microscope. The negative was used to determine the number of organisms present by projecting images of the Euglena with a film strip projector on a screen and counting the number present. This was then correlated with the absorption of light as determined by the densimeter. A series of dilutions was made and the respective densimeter readings were determined. From these readings a graph was drawn from which the population density was determined.

The culture medium was prepared and placed in test tubes and stoppered with cotton plugs. These were sterilized under 15 pounds of steam pressure for a period of 15 minutes. The cooled medium was then inoculated with a seven day old culture possessing about 250 organisms per one-tenth cubic millimeter. One loop of the sterile inoculant was introduced into fresh medium. The pH of the medium as determined by a Beckman Model H2-17 pH meter was 4.0.

The culture tubes were placed under constant fluorescent

light at a room temperature of 32° centigrade. Each tube was agitated twice daily and daily densimeter determinations were made.

On the fifth day of growth, twelve test tubes containing actively growing populations of Euglena were taken to the Civil Engineering Annex at Iowa State University where ultrasonic irradiation was performed. A Brinkman ultrasonic disperser, nebulizer, machine was used. The megacycle piezoelectric oscillator maintained an intensity output of 95 milliamps. The irradiation time was constant, five minutes.

The cavitation chamber was partially filled with distilled water and the inoculated tubes suspended in this water. Each culture was thoroughly agitated before immersion into the cavitation chamber for irradiation. The sonic generator operated continuously from the time the first of twelve cultures were irradiated until the final irradiations were completed. Because of this, the temperature of the cultures at the time of irradiation varied from 37° to 42° C.

The irradiated tubes were returned to the controlled environment where they were maintained except when the populations of Euglena were determined. The controls were treated similarly except no irradiation was applied.

## CHAPTER IV

### DATA AND INTERPRETATION OF DATA

The number of organisms per unit volume was checked by hemocytometer counts against the readings of the densimeter to develop a population density curve. Serial dilutions were made with mass cultures, and a correlation curve was developed for later population counts. The densimeter readings were correlated with the actual population count by using a hemocytometer, a microscope, and a camera. At least six photographs per ohm reading were taken, but typically ten to twelve photographs were used. The negatives were read with the aid of a film strip projector, and the average number of organisms observed were recorded opposite the ohm reading. The ohm readings and the organism numbers per 3 cubic millimeter were graphed for determining population density. A fourth degree algebraic equation was used to fit the ohm reading-density curve. Five ohm readings and their corresponding observed population numbers were selected to represent the lower portion of the curve. These numbers were between 1 and 50 and were randomly picked. The following equation resulted:

$$aX^4 + bX^3 + cX^2 + dX + e = Y.$$

The letter X referred to the ohm reading and the letter Y to

the culture density. The result of the fourth degree equation was as follows:

$$-.000680X^4 + .126541X^3 - 7.4502536X^2 + 190.884880X + 316.4395120 = Y.$$

For the ohm readings which range from 50 to 100, a quadratic equation was used to fit three numbers on the Y scale. The formula for this second degree equation is as follows:

$$ax^2 + bx + c = Y.$$

The result of the use of this formula was  $.0667x^2 + 2x + 2533 = Y$ .

For the ohm values over 100, another quadratic equation was employed, and resulted in the following values:

$$.00375x^2 + 3.5x + 3012.5 = Y.$$

Given any ohm reading it was then possible to give a mathematical population number for that reading. An ohm value graph was constructed, as shown on Figure 1, which was used to describe a population number for any one ohm reading. Points located along the graph represent the actual counts as determined with the aid of a hemocytometer.

Sterile broth culture media was inoculated and organisms grown under the conditions described in Chapter III. On the fifth day after inoculation the ohm readings on the densimeter were observed and readings were continued every 24 hours through the twenty-fourth day. After taking the

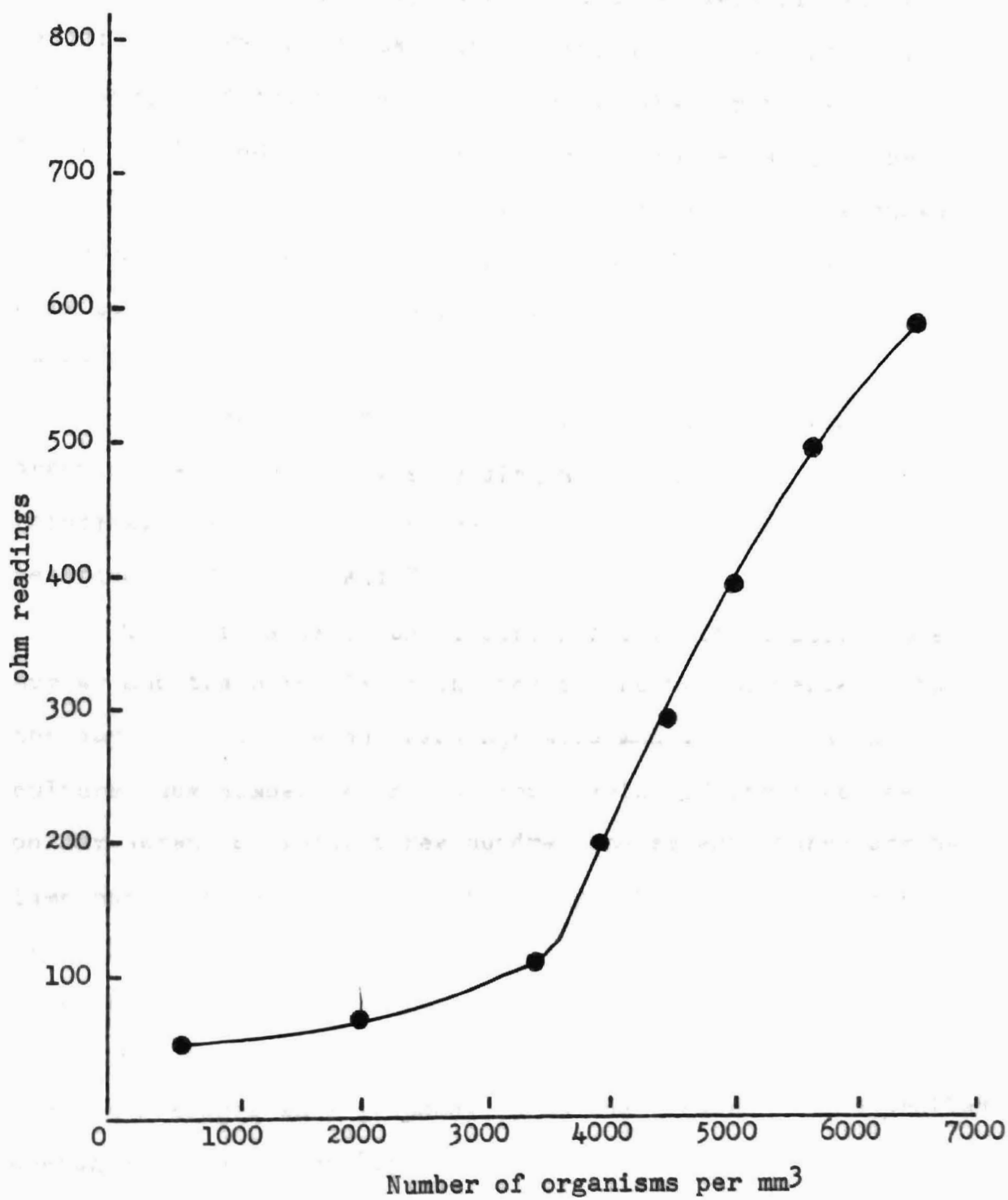


Figure 1. Graph showing organisms number per ohm reading.

readings on the sixth day, 14 culture tubes were picked at random and were irradiated with ultrasound. The remaining six tubes were maintained as controls. The first readings on irradiated and controls were taken 24 hours later. The data obtained from the 14 irradiated culture tubes are shown on Table I. Two additional series of similar experiments were performed and the results were comparable in all respects.

The average of both the control and the experimental organisms after each day's reading were determined. This information along with the real population number was also recorded on Tables I and II.

The left side of the tables refer to the culture tube number and the numerals on the top of the tables pertains to the day on which the ohm readings were made. For instance, culture tube number ten had an ohm reading of three-tenths on day seven, or about three hundred and seventy-three organisms per cubic millimeter on that day. Culture tube number seven had an ohm reading of seventy on day fourteen or about three thousand organisms per cubic millimeter.

The readings were terminated on the twenty-fourth day, and the averages were graphed. Both the treated and controlled averages are shown on Figure 2.

The ohm readings did not decrease after the ultrasound treatment, even though it was shown that the number of living

TABLE I  
DAY BY DAY OHM READINGS OF IRRADIATED EUGLENA CULTURES

Tube Number	Days from inoculation																			
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	.05	.2	.4	.6	1.5	4.3	10.5	16.5	27	70	190	200	275	300	350	500	500	600	600	600
2	.1	.4	.8	1.0	1.6	2.2	5.5	8.5	16	30	70	150	180	250	350	350	500	600	610	600
3	.1	.4	.7	.9	1.4	2.3	3.8	8.0	16	28	55	90	150	195	280	300	470	500	510	510
4	.1	.3	.4	.6	1.2	2.2	6.4	7.5	21	60	80	150	240	350	400	450	500	550	550	550
5	.1	.3	.6	1.0	2.6	5.6	14.3	16.6	45	100	160	250	350	400	450	470	500	500	500	500
6	.1	.3	.5	.9	1.8	2.8	6.7	14.0	24	60	90	190	220	300	320	340	400	500	490	500
7	.1	.4	.6	1.0	2.0	4.4	9.5	20.0	25	70	150	200	230	300	350	350	400	400	470	470
8	.1	.3	.5	.7	1.2	3.0	10.0	14.0	36	60	150	200	350	375	475	475	475	500	500	500
9	.1	.4	.7	.8	1.1	1.8	3.8	11.0	17	30	70	160	200	250	300	320	375	400	480	480
10	.05	.1	.3	.5	1.2	4.0	8.5	14.0	22	64	130	170	255	300	340	400	475	500	520	520
11	.05	.07	.2	.3	.4	1.0	2.8	7.2	20	45	65	100	200	350	400	450	500	500	500	500
12	.2	.6	1.2	2.0	3.8	12.2	20.2	50.5	90	160	200	260	290	350	450	550	550	550	550	550



TABLE I (continued)

Tube Number	Days from inoculation																			
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
13	.1	.3	.6	1.2	2.0	4.0	10.2	16.5	50	100	150	230	350	390	430	475	475	500	500	500
14	.05	.1	.4	1.0	1.8	4.0	9.6	16.0	42	180	125	190	240	350	400	450	475	520	520	510
Mean	.09	.3	.56	.89	1.7	3.8	8.7	15.7	32	70	120	181	252	319	378	420	471	509	521	520
#per mm <sup>3</sup>	333	373	421	481	620	941	1437	1925	2229	3000	3487	3759	4133	4511	4871	5144	5493	5766	5857	5847

NOTE: There are no densimeter readings for days 1-4.

TABLE II  
DAY BY DAY OHM READINGS OF CONTROL EUGLENA CULTURES

Tube Number	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	.1	.4	2.0	6.4	18.7	40	80.0	140	200	350	350	400	500	600	600	600	600	600	600	700
2	.1	.2	1.2	5.5	13.0	25	60.0	100	160	200	300	350	500	500	500	600	600	600	600	600
3	.05	.07	.4	2.2	6.1	14	27.5	40	100	180	290	350	500	500	450	500	600	600	600	600
4	.1	.4	1.2	5.2	16.0	40	60.0	95	150	200	450	450	500	500	600	500	600	600	650	630
5	.05	.07	.4	2.6	9.5	22	40.2	60	100	180	350	450	500	500	600	600	600	600	600	600
6	.1	.4	1.8	8.0	20.0	45	80.5	140	200	300	500	500	500	600	700	800	800	700	700	700
Mean.	.09	.2	1.1	4.9	13.9	31	58.0	96	151	218	373	417	500	531	575	600	633	617	625	638
#per mm <sup>3</sup>	33	345	517	1087	1685	2224	2992	3334	3677	3963	4425	5037	5710	5952	6282	6481	6752	6616	6694	6793

NOTE: There are no densimeter readings for days 1-4.

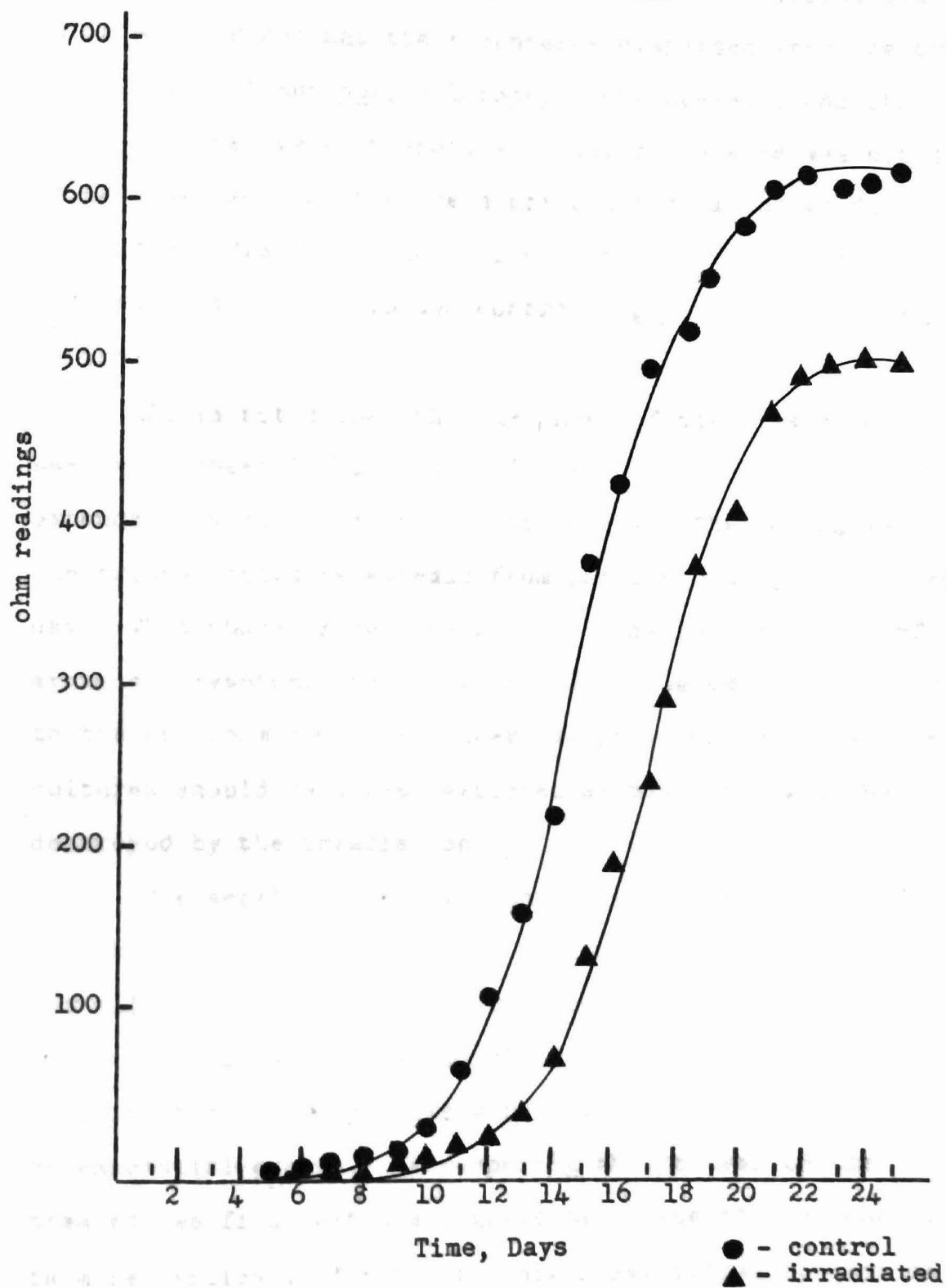


Figure 2. Graph comparing populations of irradiated cultures with control.

organisms had been drastically decreased. The irradiated cells were broken and their contents dispersed into the broth. In the case of Euglena, chlorophyll was present, and its color absorbs light, therefore no ohm difference was noted. In 48 hours most of the green color had disappeared due to chlorophyll disintegration. Increased absorption of light occurred immediately in the control organisms as recorded in Table II.

It is noted that the lag phase of the treated cultures was much longer in duration. The lag phase of the control extends from the first to the tenth day. The lag phase of the treated cultures extends from the first to the thirteenth day. This phase is defined as that time in the growth of a group of organisms when they are adjusting physiologically to the environment. The longer lag phase of the irradiated cultures should have been expected as many organisms were destroyed by the irradiation.

The accelerated-growth phase came near the end of the lag phase, and was not easily distinguished in these cultures. Not all organisms reached this phase at the same time but when they did, each organism divided at an optimal rate. At this time, the organisms were said to be in logarithmic or exponential growth. In comparing the control to the treated, we find that the logarithmic phase of the control is more vertical. The treated organisms failed to grow as

rapidly as the controls. It may be concluded that something was in the environment which curbed growth since the food source was the same in all cultures.

The deacceleration and stationary phase of the two groups show the greatest differences. The upper limit of the control group had a density of 6793 organisms per cubic millimeter. This can be interpreted in one of three ways: (1) this was the greatest density that the environment could support; (2) the food supply was depleted and growth slowed and stopped; or (3) the environment became too toxic for further growth. The upper limit of the treated group reached a density of only 5847 organisms per cubic millimeter. On the average there were 946,000 less organisms per milliliter in the irradiated cultures.

While not shown in the graph, measurements were made in an attempt to more precisely discuss the reasons the irradiated population varied from the control. One group of test tubes with only broth media were treated with ultrasound while controls were not. Both groups were then inoculated and allowed to grow for 24 days. The result of this study showed that irradiated media has no influence on the growth of Euglena. In a test of the density factor in inoculation, one group of culture tubes were inoculated and irradiated on the sixth day. The culture tubes were then inoculated with the same number of organisms that survived the irradiation.

Both the treated and controls were grown under the same conditions. The results of this experiment were similar to the previous data: the control group grew to a greater density and the experimentals plateaued at lower population levels.

A "t" test was run on the data collected to determine its significance. The results are recorded in the Appendix.

## CHAPTER V

### SUMMARY

The purpose of this study was (1) to develop methods for the determination of the effect of ultrasonics on the population growth of Euglena and (2) to describe its effect.

A densimeter was designed and constructed. Inoculated tubes of Euglena were inserted into the densimeter and the resistance of a beam of light was read on an ohm meter. The number of organisms per cubic millimeter for various ohmite readings was determined using a hemocytometer, a microscope, and a photographic apparatus.

Culture tubes were grown under constant light and at a constant temperature. On the fifth day of growth, fourteen active cultures were irradiated in an ultrasonic generator. The remaining six cultures were maintained as controls. Both the treated and the control tubes were read once a day for a period of 24 days, at which time it was impossible to distinguish the living organisms from the dead. Two additional series of similar experiments were performed resulting in comparable results.

Ultrasound had a definite effect on Euglena populations. Further study could determine more precisely the factors which caused this effect.





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APPENDIX

where  $y_1 = \log_2 x$

GROUPS.

### "T" TEST

Hypothesis: Ultrasonic sound has no effect on rate of growth of Euglena.

Alternative: Ultrasonic sound has an effect on rate of growth of Euglena.

"t" statistic would be

$$t = \frac{|\bar{X}_T - \bar{X}_C|}{S \sqrt{\bar{X} - \bar{X}}}$$

where  $H_T$ : H treated = H control

$H_A$ : H treated  $\neq$  H control

$$\bar{X}_{\text{treated}} = \frac{3824.74}{20} = 191.24$$

$$\bar{X}_{\text{control}} = \frac{6083.19}{20} = 304.16$$

Assuming that the underlying variances are equal

$$S^2_{\text{treat means}} = \frac{1}{19} \left[ \sum y_i^2 - \frac{(\sum y_i)^2}{20} \right]$$

$$S^2_{\text{control means}} = \frac{1}{19} \left[ \sum y_j^2 - \frac{(\sum y_j)^2}{20} \right]$$

where  $y_i$  = means in treated group, and  $y_j$  means in the control groups.

$$S^2 = \frac{S_T^2 + S_C^2}{2}$$

Since the variance of the two populations assumed equal, just average sample variances to obtain estimate of  $\sigma^2$  (population variance)

$$\begin{aligned} V(\bar{X}_T - \bar{X}_C) &= V(\bar{X}_T) + V(\bar{X}_C) \\ &= \frac{\sigma^2}{n} + \frac{\sigma^2}{n} \\ &= \frac{2\sigma^2}{n} \end{aligned}$$

where  $S^2 = \frac{S_T^2 + S_C^2}{2}$  is our estimate of  $\sigma^2$ .

Estimating the variance of treated group:

$$\begin{aligned} S_T^2 &= \frac{1}{19} \left[ \sum y_1^2 - \frac{(\sum y_1)^2}{20} \right] \\ &= \frac{1}{19} \left[ 1,560,734.71 - \frac{14,628,634.07}{20} \right] \\ &= \frac{1,560,734.71 - 731,431.70}{19} \\ &= \frac{829,303.04}{19} \\ &= \underline{\underline{43,645.53}} \end{aligned}$$

Estimating the variance of control group

$$\begin{aligned}
 s_T^2 &= \frac{1}{19} \left[ \sum y_j^2 - \frac{\sum y_j^2}{20} \right] \\
 &= \frac{1}{19} \left[ 3,198,735.58 - \frac{37,005,200.58}{20} \right] \\
 &= 3,198,735.58 - \frac{1,850,260.03}{19} \\
 &= \frac{1,348,475.55}{19} \\
 &= \underline{\underline{70,972.40}}
 \end{aligned}$$

$$\begin{aligned}
 s^2 &= \frac{s_T^2 + s_C^2}{2} \\
 &= \frac{43,645.53 + 70,972.40}{2} \\
 &= \underline{\underline{114,617.93}}
 \end{aligned}$$

$$s^2 = \underline{\underline{57,308.96}}$$

Now

$$t = \frac{\bar{X}_T - \bar{X}_C}{\frac{s}{\sqrt{n}}}$$

$$= \underline{191.24 - 304.16}$$

$$\sqrt{2 \left( \frac{57,308.96}{20} \right)}$$

$$= \frac{112.92}{\sqrt{5730.90}} = \frac{112.92}{75.70}$$

$$= \underline{1.49}$$

S "t" has  $2(n-1)$  difference, this equals  $2(19) = 38$ . The significant value of  $t$  for  $\alpha = .05$  is 1.96. Thus one cannot reject the hypothesis that no difference exists.

Suppose we call the difference between the two means  $S$ , then for  $t$  to be significant (keeping  $n$  fixed):

$$\frac{S}{75.70} \geq 1.96$$

$$S \geq (1.96) (75.70)$$

$$S \geq 148.37$$

That is, we would have to observe a difference of at least 148.37 before we could reject our null hypothesis, whereas we observed a difference of 112.92.

It should be noted that as the variance increases it becomes necessary to observe larger differences if it is to be significant.